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(54) Title: MICROCHAMBER FOR EMBRYO CELL CULTURE

(57) Abstract: A process for in vitro culturing of cells, an apparatus for this process and applications of said apparatus and process are provided. The cell culturing according to the invention is taking place in an especially formed receptacle (small wells formed in within a larger well) which encourages culturing and growth encompassing development, fertilization and maturing, e.g. by its small size (200-300 mm) and shape (tapered). Additionnally, a method for preparation of such a receptacle is disclosed. Finally, applications of cell culturing within the disclosed receptacles are provided.

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## MICROCHAMBER FOR EMBRYO CELL CULTURE

5 The present invention relates to a process for in vitro culturing of cells, an apparatus for this process and applications of said apparatus and process.

## BACKGROUND OF THE INVENTION

10 The in vitro culturing of cells, whether prokaryotic or eukaryotic, is of obvious interest when studying the growth, division and development of the cells per se in addition to to products resulting hereof. Various instruments and remedies are available and commercialised for cell culturing and usual devices for culturing comprise a dish or a depression in a surface that is plane and smooth. A known type of this for those skilled in the art is the so-called Petri dish. The culturing per se takes normally place within  
15 said depression or dish and the cells are usually suspended in a nutrient fluid to promote a cell development. The material used for production of these devices for culturing is usually one or more polymeric compounds such as e.g. polystyrene.

20 As to the various types of cells to culture special interest has concentrated on improvement of the in vitro culturing of eukaryotic cells including fertilisation hereof and the culturing of the embryonic cells. A part of the background for this is the wellknown interest in topics related to conditions for fertilisation in general. Therefore there is an ongoing research within optimization of the conditions for fertilization comprising both animals and humans. If the number of cells to culture is limited to a  
25 small number, as is the case for human oocytes and for animal oocytes collected for commercial purposes, the conditions for culturing are further challenged for what reason additional efforts may be needed in order to achieve maximised conditions for the culturing, including fertilization, growth and division of the cells. A number of previous investigations have published that in vitro development of preimplantation  
30 stage mammalian embryos seems to be facilitated by culturing the cells in groups, see Vajta et al. 2000.

In general by studying the prior art within conditions for cell culturing, it appears that most researchers have published results mainly focused on various biochemical topics such as e.g. optimisation of the media for culturing, the ratios of embryo/medium volume applied and options for exchange and/or change of the nutrient medium during the culturing. In contrast to this, considerations regarding the character of the physical environment per se have remained relatively disregarded so far. Thus, also within the most recent decade, very little attention has been devoted to improvements within the topic of preparing an appropriate physical environment for the cell culturing to take place. In more detail little research has been published as to the influence of the physical characteristics of the room upon the culturing of cells.

The most commonly used method when culturing few embryos in order to decrease the embryo/volume rates is to place the embryos in small droplets. By using this approach, one problem is related to fluctuations of temperature and/or evaporation of the liquid used. In order to minimize evaporation and temperature fluctuations an appropriate amount of oil is commonly used as an overlay. However, this is often accompanied with high diffusion of lipophilic material into the oil resulting in decreased culturing conditions for the embryos. Another problem is the possible accumulation of metabolic and toxic substances such as e.g. ammonia and oxygen-derived free radicals. Also the presence of these catabolics gives rise to suboptimised living conditions for the cells. A well known and frequently applied solution to this has been to culture embryos in medium sized drops and perform changes of the nutrient medium regularly, e.g. every 48 h. However, this manipulation has normally a negative and harmful impact upon embryo development.

In continuation of this, new media and incubation systems have resulted in considerable advances in the field of embryo production (Gardner et al., 1992; Takahashi and First, 1992; Gardner et al., 1994; Lane and Gardner, 1994; Trounson et al., 1994; Vajta et al. 1997b; Holm et al., 1999). It appears from these sources that both an increase in the developmental rates and much more stability in the ratios of development have been obtained. Typically, the developmental rates about 5 years ago were reported to be

within the 5-15% range, while they are now in the 40-50% range (Callesen & Holm 1999).

#### SUMMARY OF THE INVENTION

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As appears from the above, assessment in the public domain of the results of the cell culturing experiments state that developmental rate including growth and division of cultured cells is inter alia dependent upon the number of cells being assembled for culturing. Cells being assembled should here be understood as cells being in mutual contact, so that each cell is being in touch with at least one other cell. Hence, from the prior art known to the inventors it appears that the developmental rate, comprising growth rate and division rate of cells being cultured, increases when cells are assembled for culturing. The inventors have found that cells assembled in a group of cells seem to show an increase of the developmental rate within certain limits during culturing subsequently to a rise of the number of cells belonging to said group.

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It has surprisingly been shown by the present inventors, that the cell developmental rate during culturing of a relatively little number of cells increases mutatis mutandis if the cells are being cultured in wells, according to the invention. This observation is surprising and provides an improvement of the culturing conditions, when only one cell or a relatively small number of cells are to be cultured. Thus, by creating a physical space around the cells being cultured, which space forms a micro environment which is more narrow than reported in use hitherto, the cells in a relatively small number grow and develop with an increased rate than previously experienced with the size of well normally in use. Further details to this are given below in addition to a disclosure of a methodology for the preparation of said wells.

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#### DETAILED DESCRIPTION OF THE INVENTION

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In a first aspect of the present invention the dependence of the physical environment upon the in vitro growth of cells is featured. According to the invention cells are to be understood as comprising prokaryotic as well as eukaryotic cells. By assessing

published results from in vitro cell culturing experiments it appears that culturing of cells assembled in groups of a relatively small number systematically differs in terms of size of the found average developmental rate when compared to results based upon groups of cells containing a bigger number of cells. Experiments have shown that  
5       embodiments of the physical environment according to the invention especially influences the rate of growth subsequently to the phase of maturation and of fertilization.

It has surprisingly been shown by the present inventors, that the cell developmental  
10       rate during culturing of a relatively little number of cells increases mutatis mutandis if the cells are being cultured in wells, which are smaller than used previously. This observation is surprising and provides an improvement compared to the prior art within culturing conditions, when only one cell or a relatively small number of cells are to be cultured. Thus, by creating a physical space around the cells being cultured,  
15       which space forms a micro environment which is more narrow than reported in use hitherto, the cells in a relatively small number grow and develop with an increased rate than hitherto experienced with the size of well currently in use.

The current observation through the prior art is that the developmental rate of the cells  
20       appear to be lowered when culturing a relatively small number of cells or even just one cell. It has been surprisingly found as a first aspect of the present invention that when culturing such a relatively small number of cells the in vitro rate of growth, including processes such as fertilization, division of cells and maturing, is not lowered if especially developed wells are used. Such a well, which is especially developed for  
25       culturing of one cell or more cells forms according to the invention a well placed within a larger surrounding well. A well according to the invention is termed a "well of well", or a WOW.

The terminology of these two kinds of wells are as follows: A well present within a  
30       bigger well is termed a well of a first kind and a well comprising a smaller well is termed a well of a second kind, which hereinafter is used interchangeable with a WOW (Well Of Well). A well as such is to be understood as a receptacle into a plane

surface according to the present invention. This receptacle may be the result of a mechanical process (manuable or machinable) before and/or after the manufacturing of the pertinent material. The periphery of such a well may be of any form such as e.g. circular, oval, rectangular and variations hereof. The depth should be so deep as to allow space for the cells. The well should keep the cells within the well during the practical operations performed.

Culturing and growth of a cell are used interchangeable and is to be understood as to comprise all aspects and processes and results of processes during life cycles of cells including eggs such as e.g. development, fertilization and maturing.

Furthermore, the present inventors have surprisingly found that the developmental rate of the cells during the in vitro culturing of a relatively small number of cells increases if the WOW is constructed in one way when culturing only one single cell and in a different way when culturing more than one cell. According to the invention the culturing of more than one cell should be performed with said cells assembled as a group of cells. When culturing such a group of cells the WOW according to the invention is larger in terms of periphery when culturing a group of cells than when culturing only a single cell. A description of the two kinds of the WOW follows below.

One characteristic of the appearance of the WOW for the in vitro culturing of a single cell is that this WOW, seen vertically with the opening of the well being upwards, has a cross-sectional area, that has a shape as the letter V or approximately as the letter V, although the bottom per se has a form, which is more or less rounded. In a preferred embodiment, the depth of this small WOW is 210-290  $\mu\text{m}$ , preferably 225-275  $\mu\text{m}$  and particularly 240-260  $\mu\text{m}$ . The inventors have found that an additional feature of significance for the appropriate functionality of this small WOW is that the surface of the inner wall of the WOW is smooth and without scratches.

The WOW for the in vitro culturing of cells assembled in a group has according to the invention preferably a depth of approximately 260-340  $\mu\text{m}$ , preferably 275-325  $\mu\text{m}$

and particularly 290-310  $\mu\text{m}$ . The vertical cross-section of the inner shape of this large WOW is rectangular or approximately rectangular and the bottom is flat. The whole surface of the well is smooth and without scratches.

5 In a second aspect of the invention a process is disclosed for the preparation of a WOW as a beneficial physical environment for the in vitro culturing of only one single cell in addition to the preparation of a WOW for the culturing of at least two cells assembled in a group.

10 A WOW according to the present invention and preferred for the in vitro culturing of a single cell is prepared as follows. A rod is used, e.g. in the form of a needle or a changeable tip for a soldering iron, made of a material, which is still solid at temperatures used for the subsequent melting process, such as e.g. ground steel. The rod surface may well have been subject to some kind of treatment leaving the surface  
15 smooth, e.g. through covering with Teflon. Said material is heated, e.g. in a flame preferably linked to a temperature controlling equipment, until the rod melts or gets soft. Following the heating, the rod is pressed slightly until an appropriate receptacle is formed into the surface of the bottom of this well, which beforehand is present in a plane surface. The material in which the well is formed usually comprises one or more  
20 polymeric compounds such as e.g. polystyrene. The melting point of this material is to be lower than the melting point of the material of the rod. Upon touching said material by the rod the material melts or softens and the rod makes a depression, i.e. a receptacle into the bottom of the well of a second kind, thus making up a WOW. After e.g. 15-20 seconds, the melted material solidifies and the rod is removed.  
25 Approximately up to 15 WOWs may be made this way in each well of a second kind and selected for this purpose. The WOWs and the wells of the second kind are then cleaned by flushing by and/or incubating with one or more solvents suitable for cleaning of the melted material such as e.g. an aqueous buffer, a serum/plasma containing solvent or a nutrient fluid medium. The pertinent solvents may be used in  
30 combination as one solvent or as single solvents.

According to the invention a WOW preferred for culturing of at least two cells assembled in a group is prepared as a WOW for culturing of a single cell except that the rod used for making the depressions in the pertinent wells has a shape as a darning needle with no needle point.

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In the following, three examples (Examples 1, 2 and 3) of this are given for eggs from cattle. Other types of cells could however also be used, e.g. eggs from other farm animals such as pigs, from laboratory animals such as mice and from human beings.

Furthermore, the number of WOWs per well may well be lower compared to the number of WOWs used for single cell culturing. This decrease is only due to practical considerations. Approximately only up to 9 WOWs are made this way in each selected well.

In the following, two examples (Examples 4 and 5) are given for the production of different numbers of WOWs per well, and one example (Example 6) is given for the influence of this on eggs from cattle.

## EXAMPLES

### 20 EXAMPLE 1

#### **Culturing of single embryonic bovine cells**

Oocytes from the ovaries of slaughtered cows are subjected to in vitro insemination according to conventional methods within the art (see e.g. Holm et al. 1999; Vajta et al. 1997b). The developed embryos with several cells are separated into single cells and are then cultured in wells (for comparison) and WOWs according to the invention. The embryos are organised with one single cell per well and per small and per large WOW, respectively. The cells are incubated the following 7 days in 400 µl medium covered by 400 µl paraffine oil in four-well dishes (176740, Nunc, Life Technologies AS, Roskilde, Denmark). The nutrient medium and the gaseous atmosphere in the well and WOW and above the medium during the incubation of the cells is SOFaaci (synthetic oviduct fluid medium supplemented with essential and non-essential amino



acids, 0.34 mM tri-sodium citrate (Merck 1.06448, Darmstadt, Germany), 2.77 mM myo-inositol (Sigma I 7508)) with 5% calf serum (CS; National Veterinary Laboratory, Frederiksberg Denmark) and 5% CO<sub>2</sub>, 5%O<sub>2</sub> 90% N<sub>2</sub> with maximum humidity, respectively. In a preferred embodiment the culturing takes place in a Submarine Incubation System (Vajta et al. 1997a).

As a measure for the rate of development the blastocyst per oocyte ratios are determined. The determination is performed using a stereomicroscope on day 7 and the data are analyzed by logistic regression using a generalized linear model (SAS, 1993). Data differences between the incubations are compared by the contrast function of the Genmod procedure (SAS, 1993).

Results are shown in Table 1 below in the first 3 rows. It appears that the average developmental ratio is similar when the culture has been performed in wells or in drops (32 % versus 34%), while it significantly increases when performed in WOWs (60%).

Table 1. Comparison of Day 7 blastocyst/oocyte ratios of one treatment group against the control group. Different superscripts within the same module mean significant difference (P<0.05). The four modules except for the control module are referred to in the examples 1, 2 and 3.

Module	System	No. of Replicates	Blastocysts/oocytes (%)
Single zona intact embryo (Example 1)	Well	5	37 / 114 (32) <sup>a</sup>
	Drop	5	42 / 125 (34) <sup>a</sup>
	WOW	6	102 / 168 (60) <sup>b</sup>

Five zona intact embryos (Example 2)	Well	6	162 / 332 (49) <sup>a</sup>
	Drop	7	158 / 319 (50) <sup>a</sup>
	WOW	9	213 / 347 (61) <sup>b</sup>
Single zona-digested embryo (Example 3)	Well	4	17 / 69 (25) <sup>a</sup>
	Drop	4	26 / 91 (29) <sup>a</sup>
	WOW	8	136 / 256 (53) <sup>b</sup>
Control: 40-50 embryos	Well	24	973 / 1775 (55)

## EXAMPLE 2

**Culturing of more at least two embryotic bovine cells assembled in a group**

- 5 Oocytes from the ovaries of slaughtered cows are subjected to in vitro insemination according to conventional methods within the art (see e.g. Holm et al. 1999; Vajta et al. 1997b). The developed embryos are separated into groups of five embryotic cells (=five embryos, each with several cells and are then cultured in wells (for comparison) and WOWs according to the invention and organized with 5 cells per well and per
- 10 small and per large WOW, respectively. The cells are incubated the following 7 days in 400 µl medium covered by 400 µl paraffine oil in four-well dishes (176740, Nunc, Life Technologies AS, Roskilde, Denmark). The nutrient medium and the gaseous atmosphere in the well and WOW and above the medium during the incubation of the cells is SOFaaci (synthetic oviduct fluid medium supplemented with essential and
- 15 non-essential amino acids, 0.34 mM tri-sodium citrate (Merck 1.06448, Darmstadt, Germany), 2.77 mM myo-inositol (Sigma I 7508)) with 5% calf serum (CS; National Veterinary Laboratory, Frederiksberg Denmark) and 5% CO<sub>2</sub>, 5%O<sub>2</sub> 90% N<sub>2</sub> with

maximum humidity, respectively. In a preferred embodiment the culturing takes place in a Submarine Incubation System (Vajta et al. 1997a).

5 As a measure for the rate of development the blastocyst per oocyte ratios are determined. The determination is performed using a stereomicroscope on day 7 and the data are analyzed by logistic regression using a generalized linear model (SAS, 1993). Data differences between the incubations are compared by the contrast function of the Genmod procedure (SAS, 1993).

10 Table 1 shows the results as rows 4, 5 and 6, and it appears that the average developmental ratio is similar whether the culturing has been performed in wells or in drops (49% versus 50%), while it is significantly increased when performed in WOWs (61%). Compared to single cultured embryos, an increase in developmental rate is apparent from app. 33% to the app. 50% found in this experiment.

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### EXAMPLE 3

#### **Culturing of single embryotic bovine cells without zona pellucida**

Oocytes from the ovaries of slaughtered cows are subjected to in vitro insemination  
20 according to conventional methods within the art (see e.g. Holm et al. 1999; Vajta et al. 1997b). Removal of the zona pellucida is performed by an enzymatic reaction. A preferred methodology for this proteolysis according to the invention is to incubate the embryos for 2-3 min with 5 mg/ml Pronase (Sigma) according to Peura et al. (1998) in HEPES-buffered TCM-199 medium at 35-37°C. Subsequently, the embryos are  
25 incubated for 5 min in HEPES-buffered TCM -199 medium and 10% calf serum to eliminate further pronase effect. The resulting zona free embryos are thereafter separated into single embryos, each with several cells, and are then cultured in wells (for comparison) and WOWs according to the invention. Thus, a single embryo is cultured per well and per small WOW and per large WOW, respectively. The cells are  
30 incubated the following 7 days in 400 µl medium covered by 400 µl paraffine oil in four-well dishes (176740, Nunc, Life Technologies AS, Roskilde, Denmark). The nutrient medium and the gaseous atmosphere in the well and WOW and above the

medium during the incubation of the cells is SOFaaci (synthetic oviduct fluid medium supplemented with essential and non-essential amino acids, 0.34 mM tri-sodium citrate (Merck 1.06448, Darmstadt, Germany), 2.77 mM myo-inositol (Sigma I 7508)) with 5% calf serum (CS; National Veterinary Laboratory, Frederiksberg Denmark) and 5% CO<sub>2</sub>, 5%O<sub>2</sub> 90% N<sub>2</sub> with maximum humidity, respectively. In a preferred embodiment the culturing takes place in a Submarine Incubation System (Vajta et al., 1997a).

As a measure for the rate of development the blastocyst per oocyte ratios are determined. The determination is performed using a stereomicroscope on day 7 and the data are analyzed by logistic regression using a generalized linear model (SAS, 1993). Data differences between the incubations are compared by the contrast function of the Genmod procedure (SAS, 1993).

Results are shown in table 1 and it appears that the average developmental ratio is similar when the culture has been performed in wells compared to drops (app. 27%), while it is significantly increased in WOWs (app. 53%).

#### EXAMPLE 4

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##### **Manufacture of single-cell WOWs**

For the manufacture of single-cell WOWs a needle rod or a needle-like rod is used. An ordinary sewing needle made of ground steel or its like may be used. The surface of material chosen for the rod may include various compounds to facilitate the manufacturing process. The surface may comprise compounds or constituents which make the surface smooth and/or resistant to heat. An example of such a compound is Teflon.

The chosen rod, e.g. a sewing needle made of appropriate ground steel, is heated in a gas flame for 3-6 seconds or until the needle is hot enough to melt the material (e.g. polystyrene) out of which the well is made. Following the heating the needle is pressed slightly by hand into the bottom of a well in a four-well dish (176740, Nunc,

Roskilde, Denmark). The well has an outer limit that is round-like and may form a circle. Upon touching the polystyrene immediately melts and the needle makes a depression into the bottom of the well. Such a depression is a receptacle for the cell culturing and makes up a WOW. After normally 15-20 seconds, the melted material solidifies and the needle is removed. Approximately up to 15 single cell WOWs are made this way per well.

The wells are then filled with phosphate buffered saline (PBS) + 5% calf serum (CS; National Veterinary Laboratory, Frederiksberg, Denmark) and rigorously flushed by pipetting to remove air bubbles and possible loose, toxic material arising from the melting process. PBS is subsequently replaced with SOFaaci (Holm et al. 1999) and 5% CS and incubates overnight at 39°C. After a second rigorous flushing of the wells, the medium is replaced by a new aliquot of 500 µl of SOFaaci and 5% CS, and covered by 400 µl oil and used for the culturing.

#### EXAMPLE 5

##### **Production of poly-cell WOWs**

For the manufacture of poly-cell WOWs a needle rod or a needle-like rod is used. An ordinary sewing needle made of ground steel or its like may be used. The surface of material chosen for the rod may include various compounds to facilitate the manufacturing process. The surface may comprise compounds or constituents which make the surface smooth and/or resistant to heat. As an example the surface layer may consist of or include Teflon.

The chosen rod, e.g. an ordinary sewing needle made of appropriate ground steel, is heated in a gas flame for 3-6 seconds or until the needle is hot enough to melt the material (e.g. polystyrene) of the well. Following the heating the needle is pressed slightly by hand into the bottom of a well in a radiation sterilized dish delivered from NUNC, Life Technologies AS, Roskilde, Denmark (production No. 176740: The well has an outer limit which is round-like and may form a circle. Upon touching the polystyrene immediately melts and a depression is made into the bottom of the well.

These depressions are receptacles for the cell culturing and make up the WOWs. After normally 15-20 seconds, the melted material solidifies and the needle is removed. Approximately up to 9 poly-cell WOWs are made this way per well.

- 5 The wells are then filled with phosphate buffered saline (PBS) + 5% calf serum (CS; National Veterinary Laboratory, Frederiksberg, Denmark) and rigorously flushed by pipetting to remove air bubbles and possible loose, toxic material arising from the melting process. PBS is subsequently replaced with SOFaaci (Holm et al. 1999) and 5% CS and incubates overnight at 39°C. After a second rigorous flushing of the wells,  
10 the medium is replaced by a new aliquot of 500 µl of SOFaaci and 5% CS, and covered by 400 µl oil and used for the culturing.

#### EXAMPLE 6

- 15 **Comparison of the developmental rates achieved in single and poly-cell WOWs**  
Using methods described in the previous examples 1, 2 and 3, embryos are cultured in multiple versus single WOWs per well. The cells are incubated the following 7 days in 400 µl medium covered by 400 µl paraffine oil in four-well dishes (176740, Nunc, Life Technologies AS, Roskilde, Denmark). The nutrient medium and the gaseous  
20 atmosphere in the well and WOW and above the medium during the incubation of the cells is SOFaaci (synthetic oviduct fluid medium supplemented with essential and non-essential amino acids, 0.34 mM tri-sodium citrate (Merck 1.06448, Darmstadt, Germany), 2.77 mM myo-inositol (Sigma I 7508)) with 5% calf serum (CS; National Veterinary Laboratory, Frederiksberg Denmark) and 5% CO<sub>2</sub>, 5% O<sub>2</sub> 90% N<sub>2</sub> with  
25 maximum humidity, respectively. In a preferred embodiment the culturing takes place in a Submarine Incubation System (Vajta et al., 1997a).

- As a measure for the rate of development the blastocyst per oocyte ratios are determined. The determination is performed using a stereomicroscope on day 7 and  
30 the data are analyzed by logistic regression using a generalized linear model (SAS, 1993). Data differences between the incubations are compared by the contrast function of the Genmod procedure (SAS, 1993).

Results are shown in Table 2 and it appears that the average developmental ratio is similar when the culture has been performed in single compared to multiple WOWs irrespective of the three types of cattle eggs tested.

5

Table 2. Comparison of Day 7 blastocyst/oocyte ratios in different modules using multiple and single WOW per well.

Module	System	Replicate. No.	Blastocysts/oocytes (%)
Single zona intact embryo	Multiple WOW	5	65 / 114 (57)
	Single WOW	5	58 / 104 (56)
Five zona intact embryos	Multiple WOW	5	90 / 155 (61)
	Single WOW	5	85 / 141 (60)
Single zona-digested embryo	Multiple WOW	5	57 / 103 (55)
	Single WOW	5	46 / 90 (51)

Differences within the same module are not significant ( $P>0.5$ ).

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## CLAIMS

1. Process for in vitro culturing of one single cell or at least two cells assembled as a group, c h a r a c t e r i s e d in that the culturing takes place in a well of a second  
5 kind, which has a form of a receptacle, which is placed in the bottom of a well of a first kind and in that the opening of the well of said second kind is smaller compared to said well of said first kind.

2. Process for culturing of one single cell according to claim 1, wherein the cross-  
10 sectional area of the well of said second kind has a shape like the letter V or approximately like the letter V and wherein the surface hereof is smooth, plane and without scratches.

3. Process for culturing of at least two cells according to claim 1, wherein the cross-  
15 sectional area of the well of said second kind has a rectangular shape or approximately a rectangular shape and wherein the surface hereof is smooth, plane and without scratches.

4. Process for culturing of one single cell according to claim 2, wherein the depth of  
20 the well of said second kind is 210-290  $\mu\text{m}$ , preferably 225-275  $\mu\text{m}$  and in particular 240-260  $\mu\text{m}$ .

5. Process for culturing of at least two cells assembled as a group according to claim 3,  
25 wherein the depth of the well of said second kind is 260-340  $\mu\text{m}$ , preferably 275-325  $\mu\text{m}$  and in particular 290-310  $\mu\text{m}$ .

6. Apparatus for culturing of one single cell or at least two cells assembled as a group,  
comprising a smooth and plane material, wherein is made a well of a second kind,  
being formed as a receptacle into said smooth and plane material, said well of said  
30 second kind being c h a r a c t e r i s e d in that one or more wells of a first kind are made into the bottom of said well of said second kind and in that the opening of the well of said first kind is smaller compared to said well of said second kind.

7. Apparatus for culturing of one single cell or at least two cells assembled as a group according to any of the preceding claims c h a r a c t e r i s e d in that the wells of the first and the second kind are present in a material comprising polymeric compounds.

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8. Application of an apparatus according to claims 6-7 for cell culturing according to claims 1-5, wherein the cultured cells are eukaryotic cells.

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9. Application of an apparatus according to claims 6-8 for cell culturing according to claims 1-5, wherein the cultured cells are taken from a group consisting of one single embryotic bovine cell, at least two embryotic bovine cells, one single embryotic bovine cell without zona pellucida, one single porcine embryo with zona pellucida and at least two porcine embryos with zona pellucida.

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10. Process for preparation of an apparatus according to claims 6-7, c h a r a c t e r i s e d in that a hot rod is pressed slightly into the bottom of a well or receptacle present in the surface of a material, which melts by touch of said rod.

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 00/00308

## A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C12M 3/00, A01K 67/027, C12N 5/02  
According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: A01K, C12M, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BOISIS, MEDLINE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9220359 A1 (MOUNT SINAI SCHOOL OF MEDICINE OF THE CITY UNIVERSITY OF NEW YORK), 26 November 1992 (26.11.92), page 3, line 6 - line 19; page 4, line 4 - page 5; page 7, line 25 - line 32, and claims 4-13	1-9
A	--	10
X	US 5449620 A (JASPAR S. KHILLAN), 12 Sept 1995 (12.09.95), see col. 2 and claims	1,2,4,5-9
X	US 4894343 A (SHINJI TANAKA ET AL), 16 January 1990 (16.01.90)	1-9
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☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

\* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
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- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

25 Sept 2000

Name and mailing address of the International Searching Authority  
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Date of mailing of the international search report

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# INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 00/00308

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
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X	US 4975377 A (MARC E. KAY), 4 December 1990 (04.12.90), column 6, line 36 - line 59; column 9 --	1,3,6-9
A	WO 9713839 A1 (PRODUCCO AB), 17 April 1997 (17.04.97), page 10 - page 14, see figures and claim 7 -- -----	1,6-8

**INTERNATIONAL SEARCH REPORT**  
Information on patent family members

01/08/00

International application No.  
PCT/DK 00/00308

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